SPECIFICATION

TITLE OF THE INVENTION

GENE ENCODING PROTEIN CAPABLE OF REGENERATING LUCIFERIN, NOVEL RECOMBINANT DNA, AND PROCESS FOR PRODUCING PROTEIN CAPABLE OF REGENERATING LUCIFERIN

TECHNICAL FIELD

The present invention relates to a gene encoding a protein capable of regenerating luciferin, a novel recombinant DNA, and process for producing a protein capable of regenerating luciferin.

BACKGROUND ART

Luciferin is a substrate of a bioluminescence enzyme, luciferase, and after emitting light as a result of luciferase reaction, is converted to oxyluciferin. ATP measurement methods using luciferase are widely used in the fields of medical science and food hygiene. However, luciferin which is used as a substrate, is expensive and the luciferase reaction is inhibited by oxyluciferin produced after reaction. Thus, removal of oxyluciferin or regeneration to luciferin will enable further development of the ATP measurement methods using luciferase. A protein which is derived from a firefly and capable of regenerating luciferin from oxyluciferin has been found (U.S. Pat. No. 5814504). However, only a small quantity of the protein

can be extracted from a firefly body so that industrial application of the protein has been difficult.

Addition of such a protein capable of regenerating luciferin to the luciferin-luciferase reaction system enables improvement in durability of luminescence and reduction in the amount of luciferase and luciferin to be used.

DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a process for producing a protein capable of regenerating luciferin using a recombinant DNA to which a gene encoding the protein capable of regenerating luciferin has been inserted.

As a result of dedicated research on the above object, we have succeeded in isolating a gene which is derived from an insect belonging to the class Coleoptera and encodes a protein capable of regenerating luciferin, determining the gene structure, and obtaining a recombinant DNA by inserting a gene encoding a protein capable of regenerating luciferin into a vector DNA. Then we have completed the present invention by finding that a protein capable of regenerating luciferin can be produced efficiently by culturing a transformant or a transductant wherein the recombinant DNA is contained in a host cell.

That is, a first invention of the present invention is a gene which encodes a protein capable of regenerating luciferin by acting on oxyluciferin and D-cysteine.

A second invention of the present invention is the above gene which is derived from an organism capable of luminescence.

A third invention of the present invention is a gene which encodes the following protein (a) or (b):

(a) a protein which comprises an amino acid sequence represented by SEQ ID NO: 2;

(b) a protein which comprises an amino acid sequence derived from the amino acid sequence (a) by deletion, substitution, or addition of one or more amino acids, and is capable of regenerating luciferin.

A forth invention of the present invention is a gene which has 50% or more homology with the amino acid sequence represented by SEQ ID NO: 2 and encodes a protein capable of regenerating luciferin.

A fifth invention of the present invention is a novel recombinant DNA which is characterized in that the above gene encoding a protein capable of regenerating luciferin is inserted into vector DNA.

A sixth invention of the present invention is a transformant or a transductant which comprises the above recombinant DNA.

A seventh invention of the present invention is a process for producing a protein capable of regenerating luciferin which comprises culturing the above transformant or transductant in

a medium and collecting the protein capable of regenerating luciferin from the culture product.

Hereinafter, the present invention is described in detail.

The gene of the present invention which encodes a protein capable of regenerating luciferin is obtained from a Coleoptera.

For example, the gene of the present invention which encodes a protein capable of regenerating luciferin can be obtained as follows.

First, mRNA is extracted from the luminous organ of an American firefly.

Next, synthetic DNA is prepared based on an amino acid sequence of a purified protein capable of regenerating luciferin and the codon frequency of an American firefly. Then a reverse transcription polymerase chain reaction (hereinafter abbreviated as a RT-PCR method) is performed using the mRNA obtained above as a template, thereby obtaining DNA encoding a part of the protein capable of regenerating luciferin.

cDNA is synthesized from the mRNA obtained above using reverse transcriptase. Then the cDNA, as an intact cDNA or as an amplified gene encoding a protein capable of regenerating luciferin by the PCR method, is incorporated into a vector DNA by standard techniques. Examples of a vector DNA used herein include a plasmid DNA, such as pUC19(Takara Shuzo), pBR322

(Takara Shuzo), pBluescript SK+ (Stratagene), and pMAL-C2 (NEW England Labs), and bacteriophage DNA, such as λ ENBL3 (Stratagene) and λ DASH II (Funakoshi). The obtained recombinant DNA is transformed or transduced into, for example, *Escherichia coli* K-12, preferably *Escherichia coli* JM109 (Toyobo), DH5α (Toyobo) or XL1-Blue (Funakoshi), thereby obtaining transformants or transductants, respectively. In addition to the above, examples of a host cell used herein include bacteria, such as *Escherichia coli* other than *E. coli* K-12, yeast, mold, Actinomycetes, silk worms, and animal cells.

Transformation can be performed by, for example D. M. Morrison's method (Method in Enzymology, 68, 326-331, 1979). Transduction can be performed by, for example B. Hohn's method (Method in Enzymology, 68, 299-309, 1979).

A novel recombinant DNA which is purified from the above transformant or transductant can be obtained by, for example, P. Guerry et al.'s method [J. Bacteriology, vol. 116, 1064-1066 (1973)] and D. B. Clewell's method [J. Bacteriology, vol. 110, 667-676 (1972)].

Further, the entire nucleotide sequence of a gene which encodes a protein capable of regenerating luciferin is analyzed (see SEQ ID NO: 1) using DNA comprising the above gene which encodes the protein capable of regenerating luciferin and a 373A DNA sequence system (Perkin-Elmer) indicated in the later described Example (9). Then, the primary sequence of amino acids of a polypeptide which is translated by a gene comprising the

above nucleotide sequence is determined (see SEQ ID NO: 2).

Further, the present invention encompasses any gene which encodes a protein capable of regenerating luciferin comprising an amino acid sequence which is derived from the amino acid sequence of SEQ ID NO: 2 by deletion, substitution, or addition of one or more, preferably several amino acids and is capable of regenerating luciferin.

Furthermore, the present invention encompasses any gene which encodes a protein having a 50% or more homology with the amino acid sequence of SEQ ID NO: 2 and capable of regenerating luciferin.

Any method can be employed to obtain a gene which encodes a protein capable of regenerating luciferin comprising an amino acid sequence which is derived from the amino acid sequence of SEQ ID NO: 2 by deletion, substitution or addition of one or more amino acids and is capable of regenerating luciferin. Examples of such a method include site-directed mutagenesis which is a known technique to cause point mutation or deletion to occur in a gene, a method which involves selective cleavage of a gene, removal or addition of a selected nucleotide, and ligation of the gene, and an oligonucleotide mutation induction method.

A protein capable of regenerating luciferin can be produced as described below using a transformant or a transductant capable of regenerating luciferin obtained as described above, for example, a strain belonging to the genus *Escherichia*. The above

microorganism may be cultured by a normal solid culture method, preferably a liquid culture method.

A medium used for culturing the above microorganism is supplemented with, for example, one or more types of nitrogen source, such as yeast extract, Peptone, meat extract, corn steep liquor, or exudates of soybean or wheat koji; and one or more types of inorganic salt, such as potassium dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium sulfate, ferric chloride, ferric sulfate or manganese sulfate, and if necessary, appropriately supplemented with carbohydrate material, vitamin and the like.

The initial pH of a medium is appropriately adjusted to 7 to 9. Culturing is performed at 30℃ to 42℃, preferably at around 37℃ for 6 to 24 hours by aeration (agitation) - submerged culture, shaking culture, stationary culture or the like. After culturing, a protein capable of regenerating luciferin can be collected from the culture product by normal techniques for collecting enzymes.

Cells were isolated from the culture product by a technique, such as filtration or centrifugation, and washed. The protein capable of regenerating luciferin is preferably collected from cells. In this case, intact cells can be used. Preferably, the protein capable of regenerating luciferin is collected from cells by, for example, a method which disrupts cells using various disruptive means, such as an ultrasonicator, french press or Dyno-Mill, a method which digests cell walls using a cell wall

digesting enzyme, such as lysozyme, and a method which extracts enzyme from the cell using a surfactant, such as Triton X-100.

The protein capable of regenerating luciferin can be isolated from the thus obtained crude solution of protein having ability to regenerate luciferase by a standard technique for enzyme purification. Preferably performed is an appropriate combination of such techniques including ammonium sulfate salting out technique, precipitation technique using organic solvents, ion exchange chromatography, gel filtration chromatography, adsorption chromatography and electrophoresis.

The obtained protein capable of regenerating luciferin can regenerate luciferin by acting on oxyluciferin and D-cysteine.

(Method for measuring ability to regenerate luciferin)

(Reagent)

A 0.1 mM oxyluciferin

B 0.01 mM D-cysteine

C 25 mM glycylglycine + 5.4mM magnesium sulfate

D 10 mM ATP (pH7.8)

E 5 mg/ml luciferase

(Procedure)

1. Prepare a mixed solution of the following reagents.

0.005 ml A

0.010 ml B

0.085 ml C

2. Add 0.01 ml of the protein solution and allow to react at 37℃ for a certain time.

3. Mix 0.01 ml of the reaction solution with 0.1 ml of C.

4. Prepare a luciferase mixed solution of the following reagents.

10 ml D

1 ml E

5. Add 0.1 ml of the mixed solution of 4 to that of 3, and then measure the amount of light emitted using a luminometer.

Best mode for carrying out the invention

Hereinafter, the present invention is described in more detail by Examples.

EXAMPLES

[Example 1]

(1) Preparation of American firefly mRNA

The tail portion of American firefly (Sigma) 10 g disrupted with a mortar and a pestle was suspended in 10 ml of ISOGEN (Wako Pure Chemical Industries, Ltd.), a reagent for extracting RNA, and then centrifuged at 2700 r.p.m. for 5 min, thereby obtaining RNA fraction. From the fraction, 0.51 mg of mRNA was obtained according to the method described in the Current Protocols in Molecular Biology (WILEY Interscience, 1989).

(2) Synthesis of primer

Approximately 10 μg of the protein capable of regenerating luciferin purified in (1) was subjected to a protein sequencer (Perkin-Elmer Corporation), so that the N-terminal amino acid sequence was determined. Further, approximately 10 μg of the

protein capable of regenerating luciferin purified in (1) was digested with trypsin. Then 6 peptides obtained with HPLC were subjected to a protein sequencer, so that the internal amino acid sequence was determined. Furthermore, the codon frequency of American firefly was examined. Based on this information, primers shown in SEQ ID NOS: 3 and 4 were synthesized by Amersham Pharmacia Biotech's entrusted custom synthesis.

(3) RT-PCR

A reaction solution was prepared to have the following composition, and a reverse transcription reaction was allowed to proceed for 30 min at 42℃. Then, denaturation was performed at 99℃ for 5 min, and then stored at 5℃.

(Composition of reaction solution)

Magnesium chloride	5 mM
*10xRNA PCR buffer	2 μl
water	8.5 μl
dNTP	1 mM each
RNase inhibitor	1 U/μl
*AMV reverse transcriptase XL	0.25 U/μl
*oligo dT adapter primer	0.125 μM
mRNA	1 μg

*manufactured by Takara Shuzo

Next, 80 μl of the reaction solution prepared to have the following composition was added to a tube in which reverse transcription had been performed. Then PCR was performed under a reaction condition for 30 cycles, each consisting of

denaturation at 94℃ for 30 sec, annealing at 62℃ for 30 sec, and elongation reaction at 72℃ for 1.5 min.

(Composition of reaction solution)

Primer (SEQ ID NO: 3)	0.2 μM
Primer (SEQ ID NO: 4)	0.2 μM
*10xRNA PCR buffer	8 μl
Magnesium chloride	2.5 mM
*Taq polymerase	2.5 Unit
Water (add water to a final volume of 80 μl)	

*manufactured by Takara Shuzo

After PCR, the reaction solution was subjected to agarose gel electrophoresis, so that a band of approximately 0.75kb regarded as a target fragment was confirmed. The band was cut out and purified with GENECLEAN II (BIO 101).

(4) Determination and analysis of nucleotide sequence of purified DNA fragment

The nucleotide sequence of the purified DNA fragment was determined and analyzed using a 373A DNA sequence system (Perkin-Elmer). Thus, an amino acid sequence which had been deduced from the determined nucleotide sequence was confirmed to comprise the previously described amino acid sequence (His Glu Thr Gln Thr Leu Tyr Phe Val Asp Thr). Thus, a partial sequence of the gene which encodes a protein capable of regenerating luciferin was confirmed to be present in the DNA fragment amplified by the above RT-PCR.

(5) Analysis of downstream region by 3'RACE

First, a primer was designed according to the above analysis for DNA sequence, and then synthesized by Amersham Pharmacia Biotech (SEQ ID NO: 5). RT-PCR was performed using the primer, the above mRNA and 3'-Full RACE CoreSet (Takara Shuzo), thereby amplifying 3' unknown region. The reaction solution was subjected to agarose electrophoresis, a DNA fragment of approximately 650 bp was purified and extracted with RecoChip (Takara Shuzo), and the nucleotide sequence was determined and analyzed using a DNA sequencer. Therefore, the 5' region of the determined nucleotide sequence was confirmed to contain a sequence being the same as that of the 3' sequence of the partial sequence of the above gene encoding a protein capable of regenerating luciferin. Further, an amino acid sequence which had been deduced from the determined nucleotide sequence was confirmed to comprise the previously described amino acid sequence (Ile Pro Asp Pro Gln Val Thr Ser Val Ala Phe Gly Gly Pro Asn Leu Asp Glu).

(6) Analysis of upstream region by 5' RACE

First, primers were designed according to the above analysis for DNA sequence, and then synthesized by Amersham Pharmacia Biotech (SEQ ID NOS: 6 to 9). RT-PCR was performed using the primers, the above mRNA and 5'-Full RACE CoreSet (Takara Shuzo), thereby amplifying 5' unknown region. The reaction solution was subjected to agarose electrophoresis, a DNA fragment of approximately 400 bp was purified and extracted with RecoChip (Takara Shuzo), and the nucleotide sequence was determined and analyzed using a DNA sequencer. Therefore, the determined

nucleotide sequence was confirmed to contain a sequence being the same as that of the partial sequence of the above gene encoding a protein capable of regenerating luciferin. Further, an amino acid sequence which had been deduced from the determined nucleotide sequence was confirmed to comprise the previously described amino acid sequence (Gly Pro Val Val Glu Lys Ile Ala Glu Leu Gly Lys).

(7) Recovery of gene fragment by RT-PCR

A translation initiation codon and a termination codon were deduced from the above three nucleotide sequences, and then the primer DNAs of the N terminal region and the C terminal region were synthesized by Amersham Pharmacia Biotech (SEQ ID NOS: 10 and 11). RT-PCR was performed using the primers and the above mRNA, and then the reaction solution was analyzed by agarose electrophoresis. As a result, a band of approximately 900 bp was confirmed. A DNA fragment contained in the band was purified and extracted with a RecoChip (Takara Shuzo), followed by digestion with restriction enzymes *Eco* RI and *Pst* I (both manufactured by Takara Shuzo). Separately, a plasmid pKK223-3 (Pharmacia) was digested with restriction enzymes *Eco* RI and *Pst* I and purified by agarose electrophoresis. This was ligated to the above purified and extracted DNA fragment, and then transformation of *E. coli* JM109 (Toyobo) was performed. The transformant strain, *E. coli* JM109 (pLRE), was deposited to Patent and Bio-Resource Center, National Institute of Advanced Industrial Science and Technology as FERM BP-6908.

(8) Confirmation of activity

E. coli JM109 (pLRE) cells were shake-cultured to Klett 100 at 37℃ in 10 ml of TY medium (1% bacto trypton, 0.5% bactoyeast extract, 0.5% NaCl, pH 7.0) containing 50 μg/ml ampicillin. Then, IPTG was added to a final concentration of 1mM, followed by another 4 hours of culturing. The culture solution was treated 4 times (20 sec each) using an ultrasonicator (Ultrasonicgenerator, Nissei) while cooling on ice. The solution was put into an Eppendorf tube, and centrifuged at 12,000 r. p. m. with a micro centrifuge for 10 min, thereby separating into supernatant and precipitation fractions. The supernatant was transferred to another Eppendorf tube and ability thereof to regenerate luciferin was measured by the previously described method for measuring enzyme activity. While *E. coli* comprising only a vector had 0.94 kcount/ml, *E. coli* JM109 (pLRE) had 10.58 kcount/ml and was confirmed to be capable of regenerating luciferin.

(9) Analysis of gene encoding protein capable of regenerating luciferin

Confirmation of the luciferin regenerating ability of *E. coli* JM109 (pLRE) revealed that the insertion fragment of pLRE comprised the gene of the protein capable of regenerating luciferin. Then, the nucleotide sequence was determined for the plasmid DNA using a 373A DNA sequence system (Perkin-Elmer). The determined nucleotide sequence and an amino acid sequence of a polypeptide which is translated from the DNA sequence are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. The gene of the protein capable of regenerating luciferin had a coding region of 924 bp and encoded 308 amino acids.

INDUSTRIAL APPLICABILITY

The present invention is industrially very useful because the invention enables efficient production of a protein capable of regenerating luciferin.